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BIOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF *TOXOPLASMA GONDII* STRAINS OBTAINED FROM SOUTHERN SEA OTTERS (*ENHYDRA LUTRIS NEREIS*)

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ABSTRACT: *Toxoplasma gondii* was isolated from brain or heart tissue from 15 southern sea otters (*Enhydra lutris nereis*) in cell cultures. These strains were used to infect mice that developed antibodies to *T. gondii* as detected in the modified direct agglutination test and had *T. gondii* tissue cysts in their brains at necropsy. Mouse brains containing tissue cysts from 4 of the strains were fed to 4 cats. Two of the cats excreted *T. gondii* oocysts in their feces that were infectious for mice. Molecular analyses of 13 strains indicated that they were all type II strains, but that they were genetically distinct from one another.

Toxoplasma gondii is a ubiquitous apicomplexan that infects both mammals and birds and uses cats as the definitive host (Dubey and Beattie, 1988). *Toxoplasma gondii* has been reported from several species of marine mammals such as the northern fur seal (*Callorhinus ursinus*) (Holshuh et al., 1985), the West Indian manatee (*Trichechus manatus*) (Buergelt and Bonde, 1983), and 5 species of dolphins (DiGuardo et al., 1995). However, it has not been isolated from the tissues of these animals. Lack of isolation of the parasite in these cases precludes biological characterization and *T. gondii* strain typing (Howe and Sibley, 1995).

Since 1992 researchers at the National Wildlife Health Center (NWHC), Madison, Wisconsin have been conducting necropsy examinations on southern sea otters as part of an ongoing mortality monitoring program to define factors that may be responsible for limiting the growth of the southern sea otter (*Enhydra lutris nereis*). Animals that died along the California coastline were collected via a stranding network coordinated by the U.S. Fish and Wildlife Service and the California Department of Fish and Game with the assistance of other federal agencies and academic and private institutions. To date, 277 otters have been examined. A relatively high frequency (38.5%) of infectious disease has been documented as the cause of mortality in this population of otters (Thomas and Cole, 1996). One group of infectious agents targeted for further investigation was protozoal organisms suggestive of apicomplexans that were observed upon examination of tissue sections. From 1 January 1995 to 31 July 1997 a total of 112 carcasses were received and 67 otters were chosen as a subset on which isolation attempts of protozoa would be executed. To better characterize and identify these suspect organisms isolation attempts of protozoa from carcasses were made based on the following criteria, neurological signs present or absent in sea otters at stranding, condition of the carcass upon receipt from California, and the availability of cell cultures for inoculation. Therefore, not every animal sent for necropsy was sampled by cell culture isolation. In addition to isolation attempts, as part of an effort to understand the agent(s) involved in cases of encephalitis observed in some of these animals, brain tissues with demonstrable apicomplexan organisms were examined using rabbit anti-*T. gondii* polyclonal serum in an avidin–biotin complex immunohistochemical (ABC) test (Lindsay and Dubey, 1989). Herein we report isolation of *T. gondii* from southern sea otters, oocyst excretion in cats fed mouse brains containing these strains, and *T. gondii* strain genotyping of some of these strains. Characterization of other apicomplexan parasites is reported elsewhere as is full discussion of the lesions and disease implications.

MATERIALS AND METHODS

Cell culture

Human foreskin fibroblasts (HS68, ATCC CRL 1635, Rockville, Maryland), bovine turbinate cells (BT, ATCC CRL 1390), or cardiopulmonary arterial cells (CPA, ATCC CRL 1733) were grown in 25-cm^2 tissue culture flasks with RPMI-1640 plus 10% (v/v) fetal bovine serum (FBS with γ-globulin fraction), 2 mM L-glutamine, 1×10^4 U/ml penicillin, 1×10^4 g/ml streptomycin (GIBCO, Grand Island, New York), and 5×10^{-2} mM 2-mercaptoethanol. Once monolayers were confluent, the same media were used, except FBS was reduced to 2% (v/v) for monolayer maintenance. Cell cultures were maintained on 2% media until used. Once cell cultures were inoculated, 10% media containing FBS without γ-globulin was employed, and thereafter reduced to 2% media. Cell cultures were incubated at 37 C in a 5% CO_2–95% air environment. For mouse characterization studies, tachyzoites were harvested and enumerated using a hemocytometer as previously described (Lindsay and Blagburn, 1994).

Collection of sea otters and their tissues

Sea otters were found dead or killed because of their inability to survive due to advanced disease or trauma. The entire carcass of each animal was shipped to the NWHC on cool packs from California via overnight courier. Presence/absence of clinical disease if observed, postmortem condition of the carcass, and availability of cell cultures for inoculation were factors used to determine if isolation attempts would be conducted. On cases that were deemed suitable, brains were removed and portions of cerebrum, cerebellum, and brainstem were submitted for in vitro isolation of *T. gondii.* Portions of the heart and liver were also used in some cases. Portions of brain tissue were placed in Hank's balanced salt solution (HBSS) homogenized in a sterile warring blender, filtered through sterile cheese cloth, and used to inoculate cell cultures. Tissue samples from the heart and liver when submitted were processed as above. In some instances, tissues were digested in acid-pepsin solution (Jacobs et al., 1960), neutralized with sterile 3.3% sodium hydroxide in 2% phenol red, rinsed in sterile HBSS, and processed for inoculation of cell cultures. A 6–7-ml aliquot of homogenized or acid-pepsin-treated tissue sample was incubated on cell monolayers for 30 min to allow cell penetration by zoites. The inoculum was then re-

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TABLE I. Protocol for inoculation and results of inoculation of mice with strains of *Toxoplasma gondii* obtained from southern sea otters.

Strain	No. inoculated/ no. died*	Days treated†	Day PI examined‡	No. examined/ no. positive tissue cysts§
0729	4/2	6–7	74	2/2
0761	4/0	9–14	75	4/4
0790	4/0	12–15	68	4/4
0791	4/1	9–14	75	3/3
0385	4/0	None	74	4/4
0293	4/0	None	71	4/4
0335	4/0	12–15	68	4/4
0382	4/0	None	71	4/4
0374	4/0	14–17	71	4/4
0379	4/0	11–14	67	4/4
0337	4/0	9–14	75	4/4
0211	4/0	9–14	75	4/4
0226	4/0	11–13	67	4/4
0513	4/0	None	62	4/4
0623	4/0	None	62	4/4

* Number of mice inoculated/number of mice that died.
† Days postinoculation (PI) that mice were treated with sodium sulfadiazine.
‡ Day PI that mice were killed and their brains examined for tissue cysts.
§ Number of mice examined/number of mice with tissue cysts.

moved, the monolayers rinsed with HBSS, and maintenance media were added as described above. For all isolation attempts, HS68 cells were used. In addition, CPA and BT cultures were used when available. Cell cultures were maintained a minimum of 45 days postinoculation (PI) and examined twice per week for growth of tachyzoites.

Laboratory animals

Adult, female, Swiss-Webster mice, 20–25 g, were housed in cages and provided water and standard laboratory animal chow ad libitum.

Five 10–12-wk-old, female, barrier-reared domestic short-haired cats (Harlan Sprague Dawley, Indianapolis, Indiana) were housed separately in stainless steel racked cages at the NWHC. Water and standard kitten chow were provided ad libitum.

Infection of animals

Fibroblast cell cultures infected with 15 suspected strains of *T. gondii* from southern sea otters were shipped by overnight courier to the United States Department of Agriculture, Parasite Biology and Epidemiology Laboratory (PBEL), Beltsville, Maryland for mouse infectivity studies. At the PBEL, the flasks were scraped and cells inoculated subcutaneously (s.c.) into separate groups of 4–8 female Swiss Webster mice of 25 g (Tactonic Farms, Germantown, New York). No attempts to count zoites in the inocula were done, but all flasks had visible zoites. Some groups of mice were treated with 1 mg/ml sodium sulfadiazine in the drinking water for 3–11 days after clinical signs suggestive of toxoplasmosis were observed/and or deaths of cage mates (Table I). Mice were bled 36–70 days PI. The sera from each mouse was examined via the modified direct agglutination test (MAT) (Dubey and Desmonts, 1987) at dilutions of 1:25, 1:50, and 1:500 for antibodies to *T. gondii*. Any mice that died were examined for *T. gondii* by impression smears of liver and lung or by brain squash (Dubey and Beattie, 1988). Surviving mice were killed by cervical dislocation 62–75 days PI and their brains examined via squash preparation for tissue cysts.

Inoculation and examination of cats: Two Swiss-Webster mice (Harlan Sprague Dawley, Indianapolis, Indiana) each were inoculated s.c. with cell culture-derived tachyzoites isolated from 4 sea otters (strain 0226 cell culture passage 1, strain 0729 cell culture passage 18, strain 0761 cell culture passage 11, and strain 0791 cell culture passage 13) at the NWHC. Mice were given sodium sulfadiazine 1 mg/ml in the drinking water ad libitum starting day 3 PI to prevent potential deaths from acute toxoplasmosis. Mice were killed by cervical dislocation 21 days (strain 0226) and 230 days (strains 0729, 0761, 0791) PI and brains examined via squash preparation for tissue cysts (Dubey and Beattie, 1988). Cats were fed mouse tissues as follows: cat 1 was fed 1 brain and part of the liver of 1 mouse infected with strain 0226, cat 2 was fed 2 brains from mice infected with strain 0729, cat 3 was fed 1 brain from a mouse infected with strain 0761, and cat 4 was fed 1 brain from a mouse infected with strain 0791. Cat 5 was not fed infected mouse tissues. Cats were observed 5 min following feeding and once again 30 min later for evidence of regurgitation. Feces of cats were collected from days 3 to 11 PI from cat 1 or days 3 to 24 PI from cats 2 through 5. Cat feces were examined by flotation using Sheather's sugar flotation and visual presence or absence of *T. gondii* oocysts was recorded. Additional portions of the feces were processed in 2% sulfuric acid and aerated to cause sporulation of oocysts as previously described (Dubey and Beattie, 1988). Oocysts were incubated in 2% sulfuric acid for up to 68 days at 25 C and then neutralized in 3.3% sodium hydroxide in 2% phenol red. Two mice each were inoculated as follows: 1 intraperitoneally and 1 s.c. with the oocyst suspensions. Eight- or 10-wk PI mice were anesthetized, bled, and killed as described above. A minimum of 2 squash preparations from each cerebral hemisphere were examined for tissue cysts. Lung and liver impression smears and brain squash smears were conducted on mice that died prior to 8–10 wk PI. Serum samples were obtained from cats pre- and postinfection on days 0 and 28 (cat 1) and day 25 (cats 2–5) for detection of *T. gondii* antibodies via the MAT. Serum was collected from mice inoculated with cat feces-derived oocyst suspensions 62–75 days PI and examined for antibodies via the MAT.

Characterization of *T. gondii* genotypes

The genotypes at the *SAG1* and *SAG2* loci were determined by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) as described by Howe and Sibley (1995). For genetic fingerprint analysis, *Pst*I-digested genomic DNAs were resolved by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the repetitive BS probe as described previously (Howe and Sibley, 1994).

Histopathology

Sea otter brains were removed from the carcass and fixed in neutral buffered formalin. For general screening purposes, a minimum of 3 and up to 8 transverse sections of brain 7 μm thick, measuring approximately 2 × 3 cm each, were stained with hematoxylin and eosin and examined by light microscopy. Brain sections from each otter included the level of the thalamus, hippocampus, and the brainstem with cerebellum. For each animal all sections were completely scanned at a total magnification of ×40 to assess the distribution and severity of inflammation, and at least 1 section was completely scanned at a total magnification of ×100. Inflammation was scored 0–4 depending on severity.

RESULTS

Fifteen strains of *T. gondii* were obtained from 15 individual southern sea otters from the subset of 67 animals (Table I). Collection information for these 15 animals is in Table II. All but 1 strain was cultured from undigested brain tissue inoculated onto HS68 cells. Strain 0293 was cultured from heart digest inoculated onto HS68 cells. All mice infected with the 15 strains had tissue cysts in brain squash preparations (Table I). All mice that survived the infections had MAT titers ≥1:500. No other apicomplexans were isolated from the subset of 67 southern sea otters.

All 4 cats inoculated with mouse tissues infected with *T. gondii* seroconverted with antibody titers ranging from 1:200 to 1:800 in the MAT. Oocysts were seen in the feces of 2 cats (cat 1 fed isolate 0226 and cat 2 fed isolate 0729) on days 6–9 PI. Oocysts collected from these cats were infectious for mice as demonstrated by positive brain squash preparations and titers of 1:500 in the MAT.

TABLE II. Collection information on southern sea otters from which *Toxoplasma gondii* was isolated.

Sea otter	Date collected	County*	Age†	Sex‡
0729	07/07/95	SLO	A	M
0761	07/21/95	M	A	F
0790	07/29/95	SLO	A	M
0791	07/30/95	SLO	A	M
0211	04/20/96	SLO	B	M
0226	04/25/96	SLO	A	M
0293	05/31/96	M	A	M
0335	06/21/96	SLO	I	M
0337	06/23/96	M	A	M
0374	07/13/96	SLO	A	F
0379	07/17/96	SLO	B	F
0382	07/22/96	SLO	B	M
0385	07/24/96	SLO	A	F
0513	10/13/96	SC	A	M
0623	12/28/96	SLO	A	M

* SLO = San Luis Obispo; M = Monterey; SC = Santa Cruz.
† A = adult; B = subadult; I = immature.
‡ M = male; F = female.

TABLE III. Summary of histopathological findings on the brains from 15 southern sea otters from which *Toxoplasma gondii* was isolated.

Sea otter	Protozoa	Lesion score
0729	No	4
0761	No	1
0790	No	1
0791	Yes	2
0385	No	1
0293	Yes	4
0335	No	1
0382	No	1
0374	No	1
0379	Yes	2
0337	Yes	1
0211	No	1
0226	No	1
0513	No	1
0623	Yes	2

Histopathological examination of the brain tissue of the 15 otters from which *T. gondii* was isolated demonstrated at least a minimal nonsuppurative meningoencephalitis. Overall, with the exception of sea otters 0729 and 0293 that had severe meningoencephalitis, *T. gondii* was not the cause of death. In the other 13 animals inflammation was very mild and of questionable significance. In addition not all otters had *T. gondii* stages visible in tissue sections (Table III).

Genetic analysis by PCR/RFLP of 13 strains showed that they carried allele 2 at the *SAG2* locus (Table IV), thus indicating that they are all type II strains. Analysis of the *SAG1* locus demonstrated that 6 of the isolates carried allele 2, whereas 7 of the isolates had allele 3 at this locus (Table IV). Results of *Pst*I-digested genomic DNAs hybridized with the repetitive BS probe are shown in Figure 1. Although the restriction patterns or genetic fingerprints obtained for the isolates were fairly similar, several polymorphisms were seen, suggesting that the isolates are genetically distinct.

DISCUSSION

Toxoplasma gondii was isolated from 15 of 67 southern sea otters tested. Brain was the most common tissue found positive by cell culture inoculation. Successful infection of cats was demonstrated by seroconversion of all 4 cats and oocysts in the feces of cat 1 and cat 2. This successful infection of the definitive host demonstrates that some isolates from marine mammals retain their potential for oocyst production and movement of the organism back into a nonmarine cycle. Two possibilities exist as to why oocysts were not demonstrable in cat 3 fed isolate 0761 or cat 4 fed isolate 0791. It is possible that oocyst numbers were low and that laboratory methods were not sufficient to detect the low number of oocysts excreted by these cats. Another possibility is that these isolates (0761 and 0791) have lost the ability to produce oocysts in cats.

Not all animals had protozoa or significant inflammation upon histopathological analysis. This is not an uncommon phenomenon in intermediate hosts of *T. gondii.* Investigation into the role that other apicomplexan parasites might play in the genesis of inflammation and death in the sea otter population at large is still under investigation.

Only 13 isolates were analyzed genetically because the last 2 isolates were recovered late in the year and were not included. The genetic analyses revealed that the 13 strains were type II strains. These strains are associated with the majority of human toxoplasmosis cases (Howe and Sibley, 1995; Howe et al., 1997), but it is as yet unknown whether this is due to high prevalence of these strains or an increase in their capacity to cause disease. In at least 1 population of domestic animal (pigs), the type II strains accounted for about 84% of the recovered *T. gondii* strains (Mondragon et al., 1998) but were about equally as prevalent as type III strains in a survey conducted on tissues from domestic and wild animals from various sources. Howe and Sibley (1995) hypothesized that strain type

TABLE IV. Genotypes of *Toxoplasma gondii* strains isolated from southern sea otters.

		Locus	
Strain	Source	*SAG1*	*SAG2*
RH	(Type I, human)	1	1
Ptg	(Type II, sheep)	2	2
Ctg	(Type III, cat)	2	3
0729	Otter	2	2
0761	Otter	2	2
0790	Otter	2	2
0791	Otter	2	2
0385	Otter	2	2
0293	Otter	2	2
0332	Otter	3	2
0382	Otter	3	2
0379	Otter	3	2
0337	Otter	3	2
0211	Otter	3	2
0374	Otter	3	2
0226	Otter	3	2

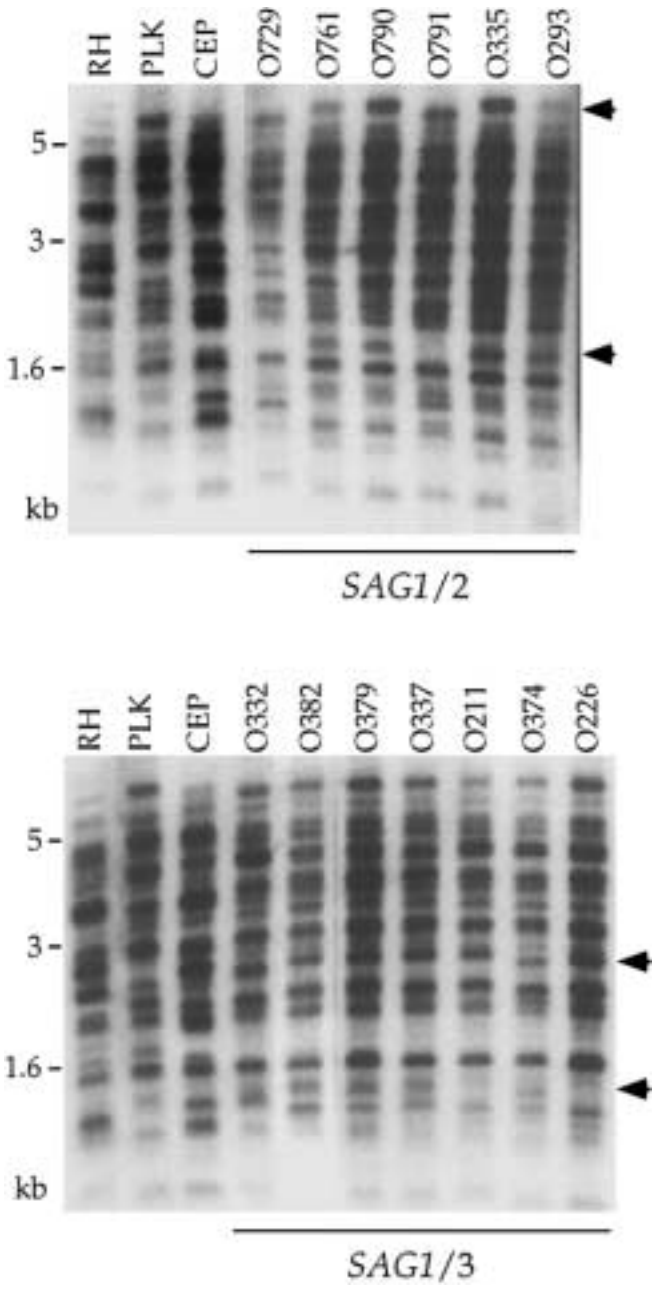

FIGURE 1. RFLP patterns for the 13 strains of *Toxoplasma gondii* from southern sea otters analyzed with the repetitive BS probe. The arrowheads indicate regions on the blots that show polymorphic bands. The RH, Ptg, and Ctg strains represent the type I, type II, and type III lineages, respectively.

II is more likely to cause disease and is not simply the most prevalent strain.

Collectively, the genetic analyses demonstrate that the strains do not represent a recent disease outbreak caused by a single parasite clone with increased pathogenicity. The presence of 2 different alleles at *SAG1* indicates that the isolates have originated from at least 2 different sources. Although the distinct genetic fingerprints shown in Figure 1 provide further evidence that the isolates do not represent a clonal outbreak, the similarity of the RFLP patterns may suggest that the isolates are fairly closely related to one another. This is also supported by the number of strains (7) with allele 3 at *SAG1*, as this is an uncommon allele that was found at a frequency of only 0.047 in a previous study of 106 strains (Howe and Sibley, 1995). This allele would not be expected in over 50% of the isolates if they were being introduced into the area from multiple outside sources.

Mammals can become infected through consumption of oocysts, tissue cysts, or by vertical passage from mother to offspring. Infection of southern sea otters most likely occurs from consuming the oocyst stage because the southern sea otter's diet of primarily invertebrates would limit exposure to tissue cysts. Cat feces containing oocysts could be entering the marine environment through storm run-off or through sewage because cat feces are often disposed of down toilets by cat owners. The role of domestic or feral cats in contaminating the beach environment with oocysts has not been investigated. However, it is documented that areas of California have large feral cat populations that are encouraged by local organizations in and around urban centers on public lands (Roberto, 1995). It is documented that a single cat can shed from 2 to 20 $\times$ 10^6 oocysts per day that can survive up to 18 mo in the environment (Frenkel et al., 1975). Given the large number of oocysts that can be shed by 1 cat and the fact that there is potentially a large number of cats in some areas, the number of oocysts contaminating beach soils could be substantial. The survival of *T. gondii* in water and maintenance of infectivity has been well documented (Mullens, 1996; Bowie et al., 1997). Consumption of oocysts by southern sea otters could be direct from contaminated water or through the consumption of invertebrates that act as phoretic agents.

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